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Nelfinavir Inhibits Maturation and Export of Herpes Simplex Virus 1

Nene N. Kalu,[a,b] Prashant J. Desai,[a] Courtney M. Shirley,[a] Wade Gibson,[b] Phillip A. Dennis,[a,b] Richard F. Ambinder[a,b]

Sidney Kimmel Comprehensive Cancer Center[a] and Department of Pharmacology and Molecular Sciences,[b] Johns Hopkins University, Baltimore, Maryland, USA

ABSTRACT

Nelfinavir (NFV) is an HIV-1 protease inhibitor with demonstrated antiviral activity against herpes simplex virus 1 (HSV-1) and several other herpesviruses. However, the stages of HSV-1 replication inhibited by NFV have not been explored. In this study, we investigated the effects of NFV on capsid assembly and envelopment. We confirmed the inhibitory effects of NFV on HSV-1 replication by plaque assay and found that treatment with NFV did not affect capsid assembly, activity of the HSV-1 maturational protease, or formation of DNA-containing capsids in the nucleus. Confocal and electron microscopy studies showed that these capsids were transported to the cytoplasm but failed to complete secondary envelopment and subsequent exit from the cell. Consistent with the microscopy results, a light-scattering band corresponding to enveloped virions was not evident following sucrose gradient rate-velocity separation of lysates from drug-treated cells. Evidence of a possibly related effect of NFV on viral glycoprotein maturation was also discovered. NFV also inhibited the replication of an HSV-1 thymidine kinase mutant resistant to nucleoside analogues such as acyclovir. Given that NFV is neither a nucleoside mimic nor a known inhibitor of nucleic acid synthesis, this was expected and suggests its potential as a coinhibitor or alternate antiviral therapeutic agent in cases of resistance.

IMPORTANCE

Nelfinavir (NFV) is a clinically important antiviral drug that inhibits production of infectious HIV. It was reported to inhibit herpesviruses in cell culture. Herpes simplex virus 1 (HSV-1) infections are common and often associated with several diseases. The studies we describe here confirm and extend earlier findings by investigating how NFV interferes with HSV-1 replication. We show that early steps in virus formation (e.g., assembly of DNA-containing capsids in the nucleus and their movement into the cytoplasm) appear to be unaffected by NFV, whereas later steps (e.g., final envelopment in the cytoplasm and release of infectious virus from the cell) are severely restricted by the drug. Our findings provide the first insight into how NFV inhibits HSV-1 replication and suggest that this drug may have applications for studying the herpesvirus envelopment process. Additionally, NFV may have therapeutic value alone or in combination with other antivirals in treating herpesvirus infections.

The herpes simplex virus 1 (HSV-1) virion is composed of viral DNA packaged within a capsid shell, which is surrounded by a tegument layer and a glycoprotein-rich envelope (1). Assembly of HSV-1 capsids occurs in the nucleus. Sedimentation analysis of infected cell lysates distinguishes three structures based on sedimentation profiles: A, B, and C capsids (2). C capsids, which sediment farthest, contain viral DNA and mature into infectious virions (3). B capsids contain the internal scaffold proteins but no DNA, whereas A capsids are empty and are thought to result from abortive attempts at DNA encapsidation. HSV-1 capsids are composed of six proteins: the major capsid protein (VP5/UL19), the triplex proteins (VP19C/UL38 and VP23/UL18), the small capsid protein (VP26/UL35), the internal scaffold protein (pre22a/UL26.5), and the two proteins (VP21/carboxyl end and VP24/amino end) resulting from self-cleavage of the maturational protease (pUL26/UL26) (3). Production of C capsids requires the activity of the pUL26 serine protease (4–7). The protease not only cleaves itself to release the N-terminal catalytic domain (VP24) but also cleaves the precursor scaffold proteins (pre-VP22a; pUL26.5) to release them from VP5. DNA-filled C capsids exit the nucleus by budding through the inner and outer nuclear membranes, and they acquire a coat of viral proteins referred to as the tegument prior to final envelopment (3, 8). Viral glycoproteins synthesized in the endoplasmic reticulum (ER) and modified in the Golgi apparatus and *trans*-Golgi network (TGN) are embedded in cytoplasmic membranes (9). Viral glycoproteins are essential for secondary envelopment, infectious virus production, initial attachment and entry into susceptible cells, and subsequent cell-to-cell spread (10–13). Enveloped virions are ultimately delivered to the plasma membrane via secretory vesicles (14).

HSV-1 is associated with a variety of diseases, many of which tend to be more serious in immunocompromised populations, such as transplant recipients and persons living with human immunodeficiency virus (HIV) (1, 15, 16). Antiviral therapies with nucleoside analogues, such as acyclovir, are generally effective in the treatment of HSV infections, unless resistance develops. Resistance is mediated by mutations or deletions in the viral thymidine kinase (TK) or DNA polymerase (17, 18). A recent report indicates that nelfinavir (NFV), an FDA-approved HIV-1 protease inhibitor, inhibits herpesvirus replication *in vitro*, but the mechanism remains unknown (17). Our studies were conducted to determine the stage(s) of replication affected by NFV and whether TK deletion mutants remain sensitive to NFV.

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Address correspondence to Richard F. Ambinder, rambind1@jhmi.edu.

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TABLE 1 Antiviral activity of nelfinavir[a]

NFV concn (μM)	HSV-1 titer (PFU/ml) in Vero cells	% Yield	HSV-1 titer (PFU/ml) in HFF cells	% Yield	HSV-1 titer (PFU/ml) in HFT cells	% Yield
0	$2.0 \times 10^8 \pm 0.14 \times 10^8$	100	$2.0 \times 10^8 \pm 0.36 \times 10^8$	100	$2.0 \times 10^8 \pm 0.47 \times 10^8$	100
2.5	$2.2 \times 10^7 \pm 0.18 \times 10^7$	11	$1.5 \times 10^8 \pm 0.18 \times 10^8$	76	$1.3 \times 10^8 \pm 0.32 \times 10^8$	65
5	$5.4 \times 10^6 \pm 0.95 \times 10^6$	3	$5.9 \times 10^7 \pm 1.2 \times 10^7$	29	$4.6 \times 10^7 \pm 0.20 \times 10^7$	23
7.5	$1.7 \times 10^6 \pm 0.10 \times 10^6$	1	$6.5 \times 10^7 \pm 0.29 \times 10^7$	32	$1.0 \times 10^7 \pm 0.41 \times 10^7$	5
10	$2.0 \times 10^6 \pm 0.76 \times 10^6$	1	$1.3 \times 10^7 \pm 0.48 \times 10^7$	7	$5.8 \times 10^6 \pm 0.14 \times 10^6$	1

[a] Titers of virus extracted from Vero, HFF, and HFT cells treated with 2.5 to 10 μM NFV for 24 h were determined by plaque assays on Vero cells. Results are mean values for numbers of plaques observed in triplicate samples ± standard error of the means (SEM) for three independent experiments, and % yield is the yield relative to that in infected cells incubated without drug (top row).

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MATERIALS AND METHODS

Cells, viruses, and drugs. Primary human foreskin fibroblast (HFF), telomerase-immortalized human fibroblast (HFT) (19), and Vero cells were maintained in minimum essential alpha medium supplemented with 10% fetal calf serum (Gibco-Invitrogen). Stocks of wild-type HSV-1 (strain KOS), a UL26 mutant virus encoding an inactive protease (KUL26H61E), a VP26-green fluorescent protein (GFP) fusion recombinant virus (K26GFP), and a TK deletion mutant (*dl*sactk) were prepared as previously described (20, 21). Nelfinavir mesylate (Z0013; Sigma-Aldrich, Milwaukee, WI) was dissolved in dimethyl sulfoxide (DMSO). Ganciclovir (GCV) (G2536; Sigma-Aldrich, Milwaukee, WI) was dissolved in 1 N HCl. Cells used as "no drug" controls were treated in parallel with cells receiving NFV or GCV, but using medium containing 0.07% DMSO to mimic the solvent component of NFV.

Plaque assay and cell viability. For growth inhibition assays, Vero, HFT, and HFF cells were infected with wild-type (KOS) or mutant (*dl*sactk) virus at a multiplicity of infection (MOI) of 10. After 1 h, virus-containing medium was replaced with medium containing no drug, NFV at specified concentrations, or 100 μM GCV. At 24 h postinfection (hpi), the cell cultures were subjected to 3 freeze-thaw cycles (−80°C/37°C) to release intracellular virus. Titration was performed on Vero cells overlaid with methylcellulose for 3 days and stained with 0.1% crystal violet (22). Cell viability was determined by the CellTiter-Glo assay for ATP (G-7570; Promega, Madison, WI), performed according to the manufacturer's instructions.

Metabolic labeling. Cells were infected at an MOI of 10 and maintained in methionine-free medium in the presence or absence of NFV. After 9 h, [^{35}S]methionine (Perkin-Elmer) was added to the cells, which were harvested 16 h later for immunoprecipitation or sedimentation analyses.

Immunoblotting and immunoprecipitation. For immunoblot assays, Vero and HFT cells were infected with KOS or UL26 protease-inactive mutant (KUL26H61E) virus at an MOI of 10 and maintained in medium containing no drug or 10 μM NFV. After 24 h, cells were scraped from the dish, pelleted, washed with phosphate-buffered saline (PBS), lysed with 2× Laemmli buffer (Bio-Rad, Hercules, CA), and further solubilized by boiling for 5 min at 95°C. Proteins were separated using a NuPAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system (Invitrogen), transferred to nitrocellulose membranes (Invitrogen) by using an iBlot system (Life Technologies), and detected by enhanced chemiluminescence (ECL) (GE Healthcare, Pittsburgh, PA). Rabbit polyclonal anti-peptide antibodies specific for amino acids 4 to 18 of HSV-1 pUL26 (also present in VP24) were generously provided by R. LaFemina and T. Conley (Merck Research Laboratories, West Point, PA).

For immunoprecipitation, Vero and HFT cells were infected with wild-type virus, maintained in medium containing no drug or 10 μM NFV, and metabolically labeled. Cells were harvested, lysed with RIPA buffer (sc-24948; Santa Cruz Biotechnology, Dallas, TX), and incubated with antibodies to HSV-1 glycoproteins gB (B4/B6), gC (gC pool), and gD (gD pool), generously provided by J. C. Glorioso (University of Pittsburgh). Protein-antibody complexes were recovered using protein A/G Sepharose beads (sc-2003; Santa Cruz Biotechnology, Dallas, TX) per the manufacturer's protocol and were subjected to SDS-PAGE.

Sedimentation analysis of virus particles. Cells were grown to 90% confluence in 100-mm dishes, infected at an MOI of 10, and maintained in medium alone or containing either 10 μM NFV or 100 μM GCV. Infected cells were metabolically labeled as described above. Intracellular capsids were recovered from Triton X-100-lysed infected cells and identified after sedimentation in sucrose gradients (23). Enveloped intracellular virus particles were similarly recovered, but in the absence of detergent, from lysates prepared by one cycle of freezing (−80°C) and thawing (37°C) followed by sonication (23, 24). Radioactivity was measured by liquid scintillation counting, and proteins in the fractions collected were resolved by SDS-PAGE.

Confocal microscopy. Vero or HFT cells (6×10^5) in four-well chamber slides (Labtek no. 1.5 borosilicate glass; Nalge Nunc, Naperville, IL) were infected at an MOI of 10 with HSV-1 (K26GFP) (25), overlaid with Liebowitz L12 medium (Gibco) containing no drug or 10 μM NFV, and viewed without further treatment by confocal microscopy at 16 hpi, all as previously described (19).

EM. Confluent monolayers of Vero cells (8×10^6) in 100-mm dishes were infected with KOS at an MOI of 10. Infected cells were maintained in medium with no drug or with 10 μM NFV. At 16 hpi, the cells were fixed and prepared for electron microscopy (EM) (3, 26) Samples were examined using either a Philips EM 420 or an FEI Tecnai 12 electron microscope; images were obtained with an SIS Megaview III camera (Olympus).

RESULTS

NFV inhibits HSV-1 replication. We investigated the effects of NFV on virus production in nontransformed (HFF) and transformed (HFT and Vero) cells. Cell monolayers were infected with KOS at an MOI of 10 and maintained in medium containing 0 to 10 μM NFV, added 1 h after virus adsorption. Plaque assay titrations were done to determine the amount of virus produced in a 24-h period (Table 1). Virus production at 10 μM NFV was reduced ≥90% in all three cell types, and the viral cytopathic effects (CPE) were comparably extensive by 24 hpi. The inhibitory effect of NFV at concentrations as low as 2.5 μM was also observed in plaque reduction assays performed on Vero cells infected with wild-type virus at an MOI of <1. The plaque assays showed no evidence of plaque formation in NFV-treated cultures at 3 days postinfection, suggesting that virus production was inhibited, not delayed (data not shown). The viability of uninfected cells treated with 2.5 to 20 μM NFV was >90% at 72 h posttreatment, as determined by CellTiter Glo assay (data not shown).

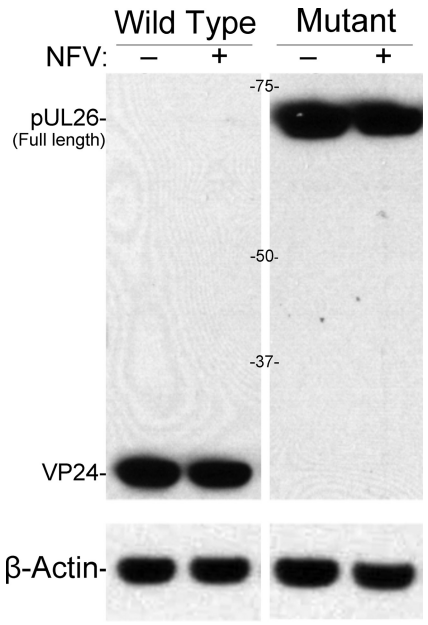

FIG 1 HSV-1 UL26 protease expression and activity are not affected by nelfinavir. Shown here is an immunoblot of Vero cells infected with wild-type virus (KOS) or protease-inactive mutant virus (KUL26H61E) that had been incubated for 24 h in the absence (−) or presence (+) of 10 μM NFV. Blots were probed with antibodies against the amino end of pUL26, stripped, and reprobed with antibodies to actin. Antibodies were detected by enhanced chemiluminescence and exposure to Kodak film. Abbreviations indicate full-length UL26 protease (pUL26) and its cleavage product, VP24. Marker protein sizes (kDa) are shown between images. HSV-1 UL26 protease expression and activity were not affected by nelfinavir.

NFV does not block production of DNA-filled capsids. Packaging of the viral DNA into preformed capsids in the nucleus and subsequent maturational events required to produce infectious virus depend on the activity of the maturational protease pUL26. To assess the effects of NFV on this process, we compared self-cleavage of the full-length protease (pUL26; 66 kDa) in the presence and absence of NFV by using an immunoblot assay able to detect both full-length pUL26 and its 26-kDa cleavage product, VP24. Cells infected with KOS or with a virus encoding an inactive protease (KUL26H61E) were cultured with or without NFV and analyzed by SDS-PAGE and immunoblotting. NFV had essentially no effect on self-cleavage of pUL26 (Fig. 1). All pUL26 was cleaved to VP24 in cells infected with wild-type virus. The amounts of VP24 produced were the same (±2%, normalized to actin) with and without NFV. In cells expressing inactive protease, pUL26 was not cleaved, and the amounts of uncleaved pUL26 were comparable (±9%, normalized to actin) with and without NFV (Fig. 1).

We then tested the effect of 10 μM NFV on capsid production by sedimentation of metabolically labeled infected cell lysates. Light-scattering bands corresponding to the three capsid forms (A, B, and C) were resolved from lysates of nontreated and NFV-treated cells (Fig. 2A). The gradients were fractionated and the three bands identified by scintillation counting (Fig. 2B). Samples from fractions 5, 8, and 10 of each gradient were subjected to SDS-PAGE. Comparable protein patterns were observed for particles from nontreated (Fig. 2C, lanes 2, 3, and 4) and NFV-treated (Fig. 2C, lanes 5, 6, and 7) cells, showing compositions characteristic of C, B, and A capsids, respectively. These results indicated that nuclear stages of virus formation, through DNA packaging to produce C capsids, are relatively unaffected by NFV. The lower level of [^{35}S]methionine radiolabeling seen in Fig. 2B for cells treated with NFV was reproducible but unexplained. Data from this and other experiments showed that actual amounts of viral protein (Fig. 1), capsids (Fig. 2A), and intracellular particles (see

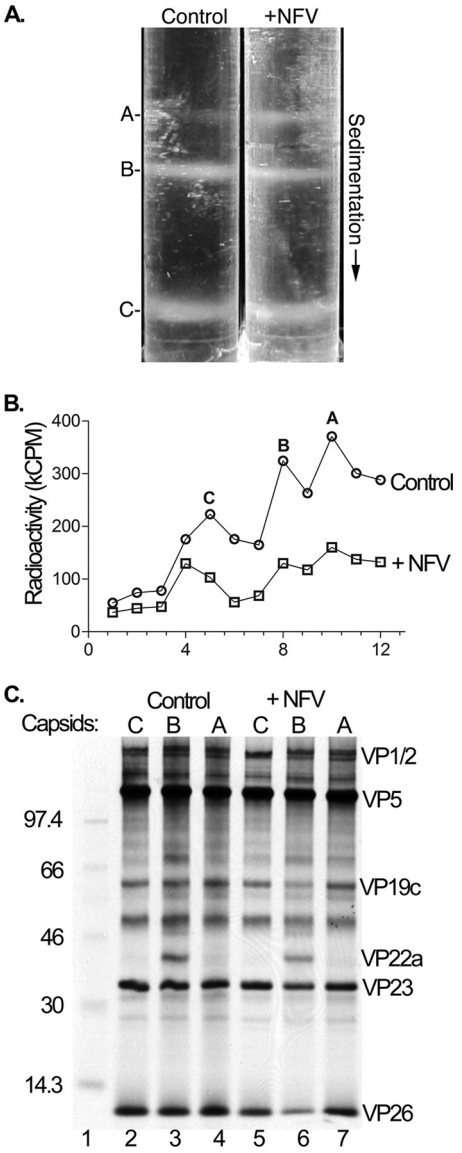

FIG 2 NFV does not alter HSV capsid production. Vero cells were infected at an MOI of 10, incubated with either no drug (control) or 10 μM NFV, and metabolically labeled with [^{35}S]methionine from 9 to 24 hpi. (A) At 24 hpi, detergent-treated whole-cell extracts were prepared and loaded onto 20 to 50% sucrose gradients. Digital pictures of the gradients show light-scattering bands corresponding to the sedimentation of A, B, and C capsids. (B) Twelve sequential fractions were collected from both gradients, and radioactivity in each fraction was measured by liquid scintillation. Corresponding peaks of radioactivity for C capsids (fraction 5), B capsids (fraction 8), and A capsids (fraction 10) were observed in the two gradients. (C) The proteins in fractions 5, 8, and 10 (C, B, and A capsids) from both gradients were precipitated with trichloroacetic acid and analyzed by SDS-PAGE. Shown here is a fluorogram of the resulting gel. Viral proteins are indicated on the right, and molecular weight markers are indicated on the left.

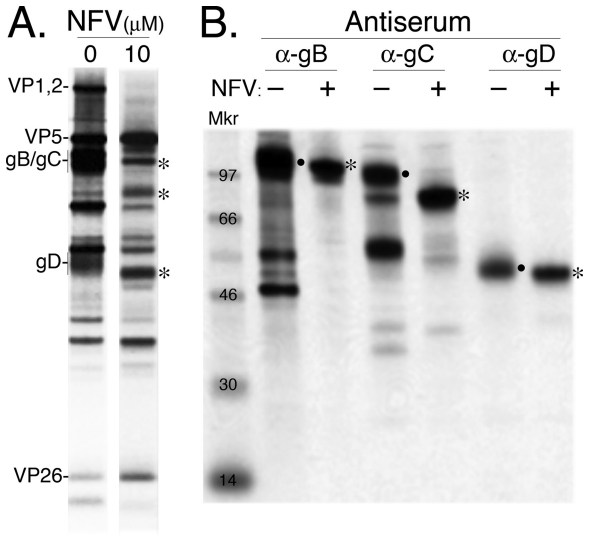

FIG 3 Tegument and glycoprotein differences detected between NFV-treated and nontreated HSV-infected cells. (A) Material was collected from the virion position of gradients containing detergent-free lysates of cells cultured with [^{35}S]methionine, with or without 10 μM NFV, and subjected to SDS-PAGE. Shown here is a fluorogram of the resulting gel. Representative capsid (VP5 and VP26), tegument (VP1,2), and envelope (gB/gC and gD) proteins are indicated. (B) Radiolabeled glycoproteins were immunoprecipitated from NFV-treated (+; 10 μM NFV) and nontreated (−) infected cells. Antisera used are indicated at the top. Dots in panel B denote fully glycosylated (mature) forms of the glycoproteins; asterisks denote proteins with electrophoretic mobilities consistent with immature gB, gC, and gD in NFV-treated cells. Proteins corresponding to immature forms of the glycoproteins are similarly indicated in panel A.

Fig. 4B) were comparable, suggesting that availability or incorporation of the radiolabeled methionine may be altered by NFV (e.g., uptake, sulfur metabolism, or protein incorporation).

Virus maturation and glycoprotein processing are inhibited in NFV-treated cells. Infected HFT cells grown with or without 10 μM NFV were metabolically labeled, lysed in the absence of detergent by freeze-thawing and sonication, and sedimented through a sucrose gradient (see Materials and Methods). A diffuse band corresponding to the expected position of enveloped nucleocapsids (virions) was observed and collected from the gradient of nontreated cells. A sample was collected from the same region of the gradient of NFV-treated cells, although no light-scattering band was visible. Analysis of the proteins detected in each sample following SDS-PAGE showed that capsid proteins (e.g., VP5 and VP26) were well represented in material from NFV-treated cells, whereas the amounts of tegument protein VP1,2/pUL36 and glycoproteins gB, gC, and gD were smaller than the amounts in the virion band from nontreated cells (Fig. 3A).

Next, we tested the possibility that NFV interferes with maturation of viral envelope glycoproteins. Monoclonal antibodies against HSV-1 gB, gC, and gD were used to immunoprecipitate each glycoprotein from lysates of [^{35}S]methionine-labeled infected HFT cells cultured in the absence or presence of 10 μM NFV. Analysis of the immunoprecipitates following SDS-PAGE showed mobility differences consistent with altered glycoprotein processing in NFV-treated cells. Electrophoretically slower fully glycosylated forms were notably reduced, and there was less evidence of presumed proteolytic degradation (e.g., smaller proteins in gB and gC lanes) in NFV-treated cells (see dots and asterisks in Fig. 3B).

Nonenveloped capsids accumulate in the cytoplasm of NFV-treated cells. The results described above indicated that cytoplasmic steps in virus formation (e.g., secondary envelopment) were more sensitive to NFV than nuclear steps (e.g., capsid assembly and DNA packaging). As a direct means of corroborating this interpretation, we used fluorescence and electron microscopies. In the first experiment, egress of capsids from the nucleus to the cytoplasm to the plasma membrane was tracked in living Vero cells by confocal fluorescence microscopy. Cells were infected at an MOI of 10 with a recombinant virus (K26GFP) tagged with GFP fused to the small capsid protein VP26 (25) and maintained in medium with or without 10 μM NFV. Confocal microscopy performed at 16 hpi showed similar patterns of fluorescence in nuclei of NFV-treated and nontreated cells (within and including the perimeter membrane; this is most obvious in Fig. 4A). Punctate structures corresponding to viral replication foci (red arrowheads) were present in both, as was a distinct ring of fluorescence at the nuclear membrane (white arrowheads). Cytoplasmic fluorescence, some diffuse and some more concentrated, was also observed in both nontreated and NFV-treated cells, but only nontreated cells showed notable fluorescence at the plasma membrane (white arrow). Similar results were seen in K26GFP-infected HFT cells (data not shown). Although there is a suggestion of somewhat weaker overall fluorescence in the NFV-treated cells, we gave greater confidence and emphasis to the striking differences evident in relative fluorescence intensity between the nuclear (stronger in NFV-treated cells) and plasma membrane (stronger in nontreated cells) regions of NFV-treated and nontreated cells (Fig. 4A).

Electron microscopy was used in a second study to determine whether DNA-containing capsids in NFV-treated cells move from the nucleus to the cytoplasm for secondary envelopment and to correlate the absence of fluorescence at the plasma membrane with the distribution of virus particles. Infected cells maintained with or without NFV were fixed and processed for thin sectioning at 16 hpi and were examined by transmission electron microscopy. No obvious difference in the population of capsids within the nucleus was noted between treated and nontreated cells (~25 total for both: 10 to 20% A, 65 to 75% B, and 10 to 15% C capsids) (Fig. 4), consistent with the findings described above (Fig. 2A, A and B capsids). Additionally, the presence of DNA-containing capsids in the cytoplasm and nucleus indicates that primary envelopment and egress from the nucleus occurred in NFV-treated cells. In marked contrast, secondary envelopment of capsids and movement out of the cell were blocked in NFV-treated cells, which showed no enveloped capsids in the cytoplasm and no mature virus in the extracellular space (~56% and 0% enveloped capsids in the cytoplasm without and with NFV, respectively, and 100% and 0% enveloped capsids in the extracellular space without and with NFV, respectively) (Fig. 4B and C).

NFV inhibits virus production in acyclovir-resistant mutants. To determine whether viral TK activity was required for NFV-mediated inhibition, plaque assays were done with a TK deletion virus (*dl*sactk). The *dl*sactk virus constructed by Coen and colleagues is resistant to inhibition by acyclovir (20). The results in Fig. 5 show that NFV inhibited virus production by both wild-type (KOS) virus and the TK deletion virus. In contrast, *dl*sactk viral replication was resistant to inhibition by ganciclovir. Taken to-

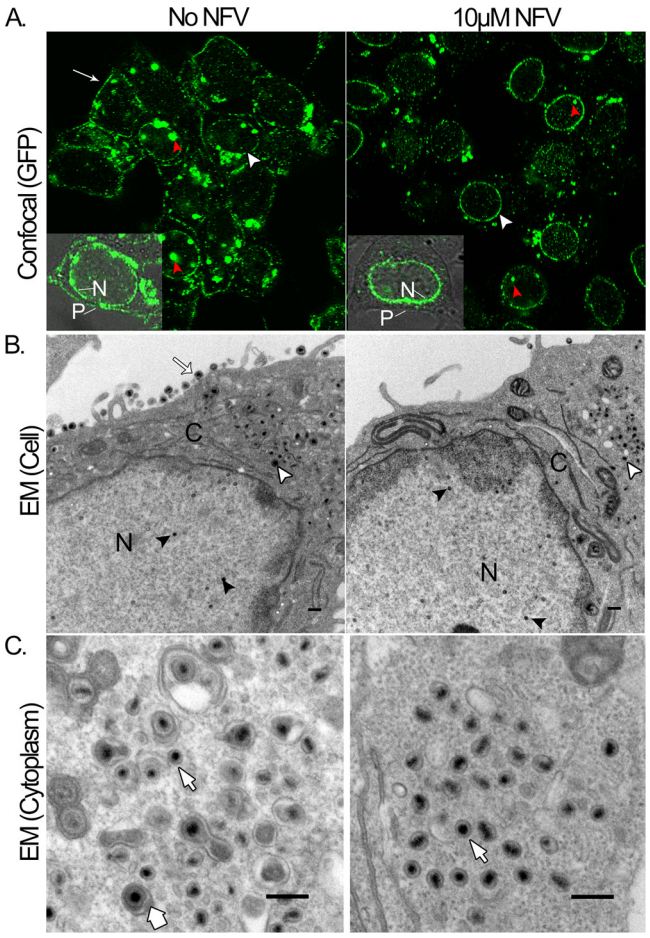

FIG 4 Nelfinavir inhibits maturation and export of virus. (A) Confocal (GFP) panels. Vero cells were infected with a GFP-expressing recombinant virus (K26GFP), incubated without (No NFV) or with 10 μM NFV for 16 h, and examined in live culture by confocal microscopy. Red arrowheads indicate viral nuclear replication sites, white arrowheads indicate the nuclear membrane, and the white arrow indicates the plasma membrane. Insets show merged GFP and phase-contrast images of single cells in nontreated and NFV-treated cells, with the nuclear (N; fluorescent in both) and plasma membranes (P; fluorescent only in nontreated cells) indicated. (B) EM (cell) panels. Vero cells infected with wild-type virus were incubated for 16 h without (No NFV) or with 10 μM NFV and then processed and evaluated by electron microscopy. Black arrowheads indicate DNA-filled capsids in the nucleus (N), white arrowheads indicate enveloped or nonenveloped capsids in the cytoplasm (C), and the white arrow indicates mature virions in the extracellular space of nontreated cells. Bars = 400 nm. (C) EM (cytoplasm) panels. Enlarged cytoplasmic views are shown. The thick arrow at the bottom indicates an enveloped capsid in a nontreated cell; thin arrows indicate nonenveloped capsids present in both nontreated and treated cells. Bars = 200 nm. Particle counts were as follows: for nuclear capsids (B), ~20 without NFV and ~20 with NFV; for enveloped nucleocapsids outside the cell (B), ~16 without NFV and 0 with NFV; for enveloped capsids in cytoplasm (C), ~15 without NFV and 0 with NFV; and for nonenveloped capsids in cytoplasm (C), ~12 without NFV and ~30 with NFV.

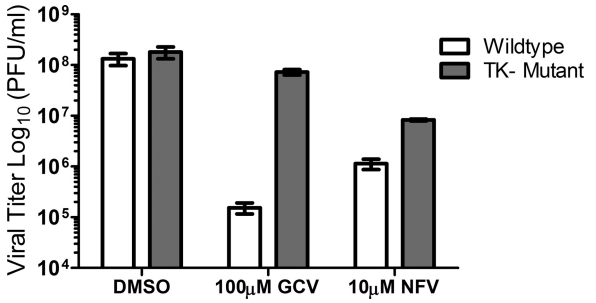

FIG 5 Absence of viral TK does not affect antiviral activity of NFV. Cells infected with either wild-type (KOS) or TK mutant (*dl*sactk) virus at an MOI of 10 were incubated without drug (DMSO), with ganciclovir (100 μM GCV), or with 10 μM NFV for 24 h. Virus yields were determined by titration on Vero cells. Results are means ± standard errors of the means (SEM) for two independent experiments.

gether, these results confirmed that the production and export of infectious virus were inhibited by NFV, even in a TK deletion mutant.

DISCUSSION

We confirmed that NFV treatment is associated with a >90% reduction in infectious HSV production, as originally reported by others (17). In that earlier report, the nature of antiviral action was not investigated. In the work reported here, we present evidence that NFV exerts its inhibitory effect on HSV at late stages of virus maturation. Capsid assembly, DNA packaging, and capsid egress from the nucleus all appear relatively unaffected by NFV, whereas secondary envelopment, glycoprotein processing, and release of infectious virus from the cell were severely impaired.

Our finding that NFV, a selective inhibitor of the HIV aspartyl protease, did not affect the HSV-1 serine protease is perhaps not surprising, but it was important to verify this finding given that the activity of this protease is essential for producing infectious virus. We demonstrated that self-cleavage of the protease precursor (pUL26) is unaffected in NFV-treated infected cells. The production of DNA-containing C capsids in NFV-treated cells constitutes additional evidence that NFV does not inhibit the HSV protease, since DNA packaging to form C capsids requires cleavage of the internal scaffolding proteins (pUL26 and pUL26.5) by the active protease. Thus, our findings provide no evidence that the inhibitory effects of NFV on viral replication involve the HSV-1 UL26 protease.

We also determined by EM that DNA-containing capsids were present in the cytoplasm of NFV-treated cells, indicating that movement of these particles from the nucleus by the primary envelopment/deenvelopment pathway was unaffected by NFV. We noted no gross differences in the integrity of the nuclear membrane that might suggest direct "leakage" or blebbing of nucleocapsids from the nucleus, which has been suggested as an alternate egress mechanism (27). Additionally, we did not observe differences in the distribution of GFP-tagged capsids associated with the nuclear membrane in NFV-treated cells that might result from altered membranes. Taken together with its lack of inhibition of the viral protease and nuclear capsid production, our data indicate little or no effect of NFV on HSV-1 replication up to and through movement of DNA-containing capsids from the nucleus into the cytoplasm, where final envelopment occurs. In contrast to the absence of obvious differences in nuclear stages of HSV replication in NFV-treated cells, differences were found in all cytoplasmic maturational events studied. The most striking of these was the absence of enveloped capsids and increased number of nonenveloped capsids in the cytoplasm of NFV-treated cells, as revealed by EM. Mutations in several herpesvirus tegument proteins (e.g., VP1,2, UL37, and VP16) and major glycoproteins (gB and

gD) result in a similar phenotype of severely impaired secondary envelopment (23, 28–32). Considered together, these observations are compatible with the possibility that NFV may interfere directly or indirectly with the correct incorporation or processing of these or other tegument and envelope proteins during virus maturation.

NFV has been reported to have multiple effects on cellular functions (33, 34). These include ER stress induction, radiation sensitization, and inhibition of site 2 protease, HSP90, phosphatidylinositol 3-kinase (PI3K)/Akt pathway, and STAT3 signaling (35–38). HIV protease inhibitors such as NFV have also been implicated in the alteration of nuclear lamin processing (39). Although the studies presented here do not identify a specific pathway dysregulated by NFV in HSV-infected cells, they focus attention on those coupled with tegument formation and secondary envelopment. NFV may have general applications in studying these processes, as brefeldin A aided in distinguishing and studying primary and secondary envelopment (40).

The effects of NFV on HSV-1 may be of clinical interest insofar as "drug-resistant" HSV-1 lacking TK activity remains sensitive to inhibition by 10 μM NFV. In addition to its approved use as part of combination therapy for the treatment of HIV infection, NFV has also been studied as an antineoplastic agent and as a radiosensitizer. Two single-agent dose-escalation trials in cancer patients have shown that a plasma level of 10 μM can be sustained in patients without dose-limiting adverse effects (41, 42). While the effects of NFV may be modest compared to those of acyclovir or ganciclovir in drug-sensitive isolates, in drug-resistant isolates, a 90% reduction in viral replication may be clinically meaningful.

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None of the authors report a conflict of interest.

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